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Influenza virus H1N1 induced apoptosis of mouse astrocytes and the effect on protein expression

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ABSTRACT

Objective: To investigate the effects of influenza A virus H1N1 infection on the proliferation and apoptosis of mouse astrocytes cells and its protein expression. **Methods:** After mouse astrocytes was infected with purified influenza A virus H1N1 *in vitro*, viral integration and replication status of the cells were detected by RT-PCR assay, cell proliferation and apoptosis was determined by MTT method and flow cytometry, respectively. Associated protein expression was detected by Western blotting. **Results:** Agarose gel electrophoresis showed H1N1 virus can infect astrocytes and can be copied. MTT staining showed H1N1 virus infection can inhibit the proliferation of mouse astrocytes, which makes cell viability decreased significantly. Flow cytometry showed that the proportion of Annexin V staining positive vascular endothelial cells in the influenza A virus group was significantly higher than that in the control group. Western blot analysis showed after 24 h and 32 h of infection, there were cells caspase-3 protein and the expression of its active form (lysed caspase-3 protein) increased. The proportion of Bax/Bcl-2 also increased. **Conclusions:** Influenza A virus can infect human vascular endothelial cells and proliferation and it can induce apoptosis of endothelial cells.

1. Introduction

In some influenza virus infection populations, there were not only respiratory diseases, but also central nervous system (CNS) diseases, such as influenza-associated encephalopathy, encephalitis[1,2]. Influenza acute encephalopathy (IAE) is the important and common influenza virus infection complication in clinic. After respiratory symptoms, patients also have severe neurological symptoms after 1 to 2 days of infection, consciousness subsided, coma, severe cases may cause neurological sequelae or death[3–5].

Astrocytes is an important source of cytokines in brain, it always accompanied the neurons development in CNS.

Astrocytes accounted for more than 50% of the total number of brain cells; it can support and nourish the neurons. Thus astrocytes can directly affect the survival of neurons, and can regulate the nervous system microenvironment by adjusting the uptake and release of glutamate, scavenging free radicals, water transportation, producing cytokines, chemokines, and NO and other functions. That plays an important role in brain homeostasis and stabilizing blood-brain barrier[6–9].

In this study, we focused on the cytogenetic response of astrocytes to influenza virus infection. We use influenza virus H1N1 to infect mice astrocytes and study the role of influenza virus on nerve cells by observing the apoptosis status of astrocytes, make clear the apoptotic signaling pathway transmission after nerve cells infected by influenza virus, and lay the foundation for future studies on the neural protection after influenza virus infection.

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2. Materials and methods

2.1. Isolation and culture of neonatal mouse cortical glial cells

Cerebral cortex tissue of 1 to 2 days newborn mice was taken and placed in DMEM/ F12 (1B1, v/v) medium according to literature. They were beat to dissociate cells, filtered with 50 μ m filter to obtain cell suspension, then placed at 37 °C for 30 min to remove fibroblasts. The remaining unattached cells were placed in 37 °C 5% CO₂ incubator and cultured. The unattached cells were removed on 2nd day and 4th days of cell culture, and medium was changed every 5–7 days, until the confluent monolayer was formed. After culture by 0.05% pancreatin digestion at 37 °C for 10 min, cells were collected and cultured centrifugal. After one day of adherent, cell culture flask was fixed on definitive orbit shaking table. The conditions were: 37 °C 200 rpm, amplitude 30 mm for 60 min, the suspended cells were discarded. They were cultured in DMEM/F12 medium containing 10% FBS for 1 d, under the shaking condition to isolate and obtain pure astrocytes^[10].

2.2. Glial cells infected with influenza virus

H1N1 influenza virus was isolated from clinical influenza case. After adherent in six–well plates, mouse astrocyte were infected with the H1N1 virus with 1 mL 4×10⁵ pfu influenza virus. Multiplicity of infection (MOI) was 2 and adsorbed at 37 °C for 1 h.

Then 2 mL serum–free DMEM/F12 medium was added in each well, they were placed in a constant temperature of 37 °C 5% CO₂ incubator. Uninfected hole was set as the negative control group. In the infection group, cells were collected after 12 h and 24 h of stimulation for subsequent trials.

2.3. Measured cell viability with MTT assay

Well developed astrocytes were seeded in 96–well cell culture plate, divided into control group (no virus) and experimental group (add virus). In each group three parallel wells were set up, added with MTT after 12 h and 24 h. They were incubated 1–4 h at 37 °C. After centrifugation, the supernatant was discarded. DMSO 100 μ L was added to each well, 96–well plates were shocked by enzyme mark instrument, absorbance value was measured at 590 nm .

2.4. Apoptotic cells was detected by flow cytometry

Astrocytes were suspended with virus infection and the

astrocytes of the control group with binding buffer, the density was adjusted to 1×10⁵/mL. Annexin V was added. CThey were centrifuged after incubation at room temperature for 15 min, washed with ice–cold PBS, resuspended in binding buffer. Apoptosis was analyzed by flow cytometry after PI staining.

2.5. Western blotting experiments

Cells were collected and cell proteins were extracted by normal cell lysing method, protein concentration was determined by BCA method, then 30 μ g protein upper sample was taken. Proteins was transferred to PVDF membrane after 10% SDS–PAGE electrophoresis, blocked with 5% fat–free milk. PRAP, cleaved–PARP, caspase–3 antibody were added for incubation, respectively. The secondary antibody was labeled by HRP and then were incubated. Electrochemiluminescence and developing photographs was performed. β –actin was used as internal control, the experiment was repeated three times.

Data were expressed as mean±SD and were analyzed by SPSS/Win15.0 software (SPSS, Chicago, IL) statistics software. One–Way ANOVA was applied and $P < 0.05$ was considered as statistical significant difference.

3. Results

3.1. Mouse astrocyte morphology

Observation of the separation and purification of mouse astrocytes under an inverted microscope showed that large nuclei which were round or oval, cells elongated with branches and irregular protrusions, most of them were radial polygon (Figure 1). The cell viability of the control group was 91.2%, while after H1N1 influenza virus infection for 12 h and 24 h, the cell viability was decreased significantly ($P < 0.05$), and was 76.5% and 54.4%, respectively.

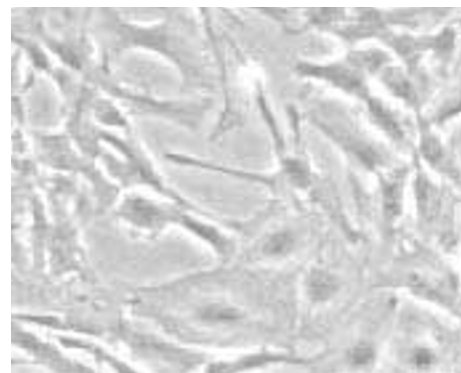


Figure 1. Purified mouse astrocytes.

3.2. Cell viability

MTT results showed that after 12 h (21.3%) and 24 h (36.1%) of H1N1 influenza virus infection of mice astrocytes, the cell viability was decreased significantly compared with untreated cells in the normal group.

3.3. Apoptotic rate

Detection apoptotic cells with flow cytometry showed that after 12h of virus infection, apoptosis rate reached 21.3%, after 24h of virus infection, the apoptosis rate was 36.1%, both were significant higher ($P<0.05$) than that of the untreated cells in the control group (3.5%).

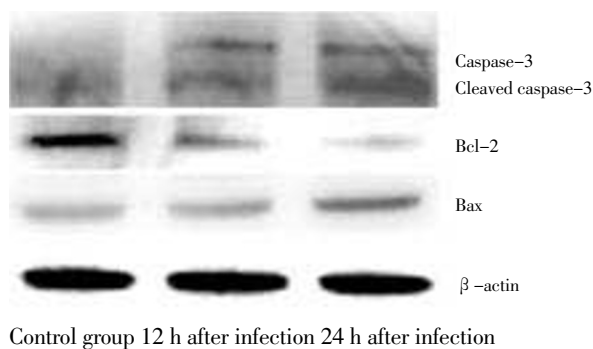


Figure 2. Western blotting detected apoptosis-related protein levels.

3.4. Apoptotic protein expression

In order to study the mechanism of influenza virus infection-induced apoptosis of astrocytes, we used western blotting experiments to detect the expression levels of apoptosis-related signaling proteins. The results showed that the influenza virus can activate Caspase-dependent apoptotic pathway and activate caspase-3, thereby inducing apoptosis. As Figure 2 showed, after 12 h and 24 h of viral infection, the apoptotic protein caspase-3 expression and its activity form cleaved caspase-3 expression were both significantly increased. In addition, we found that the pro-apoptotic protein Bax expression increased, while the inhibitor of apoptosis protein Bcl-2 expression was decreased.

4. Discussion

H1N1 flu virus infection induces serious damage on health, it also cause respiratory Disease, it also causes

central nervous system lesions. But the pathogenic mechanisms of the nervous system damage has still not been well understood, and relevant report are few[2, 10–12]. Astrocytes play an important role in nerve cell functions, glial cells infected with influenza virus is reported to cause cytokine storm effect, induce cells to release high levels of pro-inflammatory cytokines, which could lead to the corresponding immune pathological damage. Studies on the characteristics of apoptosis of nerve cells infected with influenza virus were less[13–15]. This study aimed to study the apoptosis of astrocytes stress response to influenza virus infection and to provide the basis for further studies on neuroprotection.

Apoptosis is different from necrosis. Regulation of apoptosis involve many genes. Apoptosis is not a passive process, but an initiative process, which involves a series of function associated with cell proliferation such as genes activation, expression and regulation, etc. For example there are some proto-oncogenes and tumor suppressor genes which can participate in the reproductive and apoptosis process of cells, in which more research were on Bcl-2, Fas/APO-1, c-myc, p53, ATM, *etc*[16,17].

Under physiological conditions apoptosis is strictly regulated. Caspase in normal cells are in the non-activated zymogen state. We studied the apoptosis signal pathway of mice astrocytes after H1N1 influenza virus infection, the result showed that influenza virus can rapidly activate caspase-related apoptosis signal. After the apoptotic program began, caspase is activated, the activated form protein levels increase, then apoptotic protease cascade reaction happened and induce the apoptosis[18,19]. After 12 h infection with influenza virus, we detected the glial cells and found that the expression of caspase-3 and its active form increased. Bcl-2 family anti-apoptotic members Bcl-2 and Bcl-xL are important proteins for the inhibition of apoptosis, while before the effect of apoptosis, the expression of Bax, Bad can promote cell apoptosis[20,21]. This study found after the H1N1 virus being infected with astrocytes, not only caspase-3 protein expressions increased, Bax/Bcl-2 ratio also increased indicating that virus infection can activate the corresponding apoptotic signals and cause pathogenesis injury of the nerve cells.

Our results suggest that H1N1 influenza A virus can infect astrocytes in vitro and induce apoptosis. After infection of astrocytes, apoptosis increased with time. Influenza virus infection can induce apoptosis and activate apoptotic signaling pathways. Therefore, we think that the intervention

of nerve cells apoptosis pathway may be an important way to reduce the nervous system damage caused by influenza virus infection, which needs to be further studied.

Conflict of interest statement

We declare that we have no conflict of interest.

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